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August 4, 2000

Attorney Docket No.: 07787-004003

Box Patent Application
Commissioner for Patents
Washington, DC 20231

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1855-1930

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1859-1951

Presented for filing is a new continuation patent application of:

Applicant: JOEL S. GREENBERGER AND DAVID R. HURWITZ

Title: METHODS OF PREPARING BONE MARROW STROMAL CELLS FOR USE IN GENE THERAPY

The prior application is assigned of record to ALG Company, a Massachusetts corporation, by virtue of an assignment submitted to the Patent and Trademark Office for recording on April 8, 1996 at 7882/0022.

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Enclosed are the following papers, including those required to receive a filing date under 37 CFR 1.53(b):

	Pages
Specification	34
Claims	3
Abstract	1
Declaration	2
Drawing(s)	5

Enclosures:

-- Form PTO-1449, 4 pages, and Form PTO 892, 2 pages, listing documents cited in the parent applications. Please confirm that these have been considered in this application by returning a copy of the Form PTO-1449 with the Examiner's initials.
— Preliminary amendment, 3 pages.
— Postcard.

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August 4, 2000
Date of Deposit

Matthew Morrissey
Signature

Matthew Morrissey
Typed or Printed Name of Person Signing Certificate

This application is a continuation (and claims the benefit of priority under 35 USC 120) of U.S. Application Serial No. 09/107,051, filed on June 30, 1998 (currently pending) which is a continuation of U.S. Application Serial No. 08/581,053, filed on December 23, 1997. The disclosure of the prior application is considered part of (and is incorporated by reference in) the disclosure of this application.

Preliminarily, on page 1 of the specification, between lines 4 and 5, insert --This application is a continuation of U.S. Serial No. 09/107,051, filed on June 30, 1998, (now pending) which is a continuation of U.S. Serial No. 08/581,053, filed on December 29, 1995 (now abandoned), which are both incorporated herein by reference in their entirety.--

Cancel in this application claims 12 through 20.

Basic filing fee	$690.00
Total claims in excess of 20 times $18	$0.00
Independent claims in excess of 3 times $78	$0.00
Fee for multiple dependent claims	$0.00
Total filing fee:	$690.00

A check for the filing fee is enclosed. Please apply any other required fees or any credits to deposit account 06-1050, referencing the attorney docket number shown above.

If this application is found to be incomplete, or if a telephone conference would otherwise be helpful, please call the undersigned at (617) 542-5070.

Kindly acknowledge receipt of this application by returning the enclosed postcard.

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Respectfully submitted,

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Reg. No. 32,983
Enclosures

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Joel S. Greenberger *et al.* Art Unit : Unknown
Serial No. : To Be Determined Examiner : Unknown
Filed : Herewith
Title : METHODS OF PREPARING BONE MARROW STROMAL CELLS FOR USE IN GENE THERAPY

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Please amend the application as indicated below and consider the following remarks.

In the Specification:

On page 1, between lines 4 and 5, insert the following paragraph.

-- This application is a continuation of U.S. Serial No. 09/107,051, filed on June 30, 1998, (now pending) which is a continuation of U.S. Serial No. 08/581,053, filed on December 29, 1995 (now abandoned), both of which are incorporated herein by reference in their entirety.--

In the Claims:

Cancel claims 12-20 without prejudice.

Amend claims 1 and 7-11 as follows.

1. (Amended) A method of [preparing] obtaining a preparation of bone marrow stromal cells (BMSCs), [for implantation for gene therapy, said] the method comprising:

(a) [obtaining bone marrow stromal cells;

(b) culturing the stromal cells to obtain an expanded number of cultured stromal cells;

(c)] transfecting cultured [stromal cells] BMSCs with an exogenous gene to obtain transfected [stromal cells] BMSCs; and

[(d)] (b) cryopreserving the transfected [stromal cells until implantation] BMSCs, wherein the level of expression of the exogenous gene in the transfected and cryopreserved BMSCs is comparable to the level of expression of the exogenous gene in transfected BMSCs that are not subsequently cryopreserved.

7. (Amended) The method of claim 1, wherein [said] the exogenous gene encodes a secreted peptide.

8. (Amended) The method of claim 7, wherein [said] the secreted peptide is a serum protein, a blood clotting factor, a cytokine, a lymphokine, a growth factor, a peptide hormone, a lipid binding protein, a metabolic enzyme, an antibacterial peptide, an antimicrobial peptide, an antifungal peptide, or a neurotransmitter.

9. (Amended) The method of claim 8, wherein [said] the blood clotting factor is factor VIII or factor IX.

10. (Amended) The method of claim 1, wherein [said] the exogenous gene encodes a cell surface molecule.

11. (Amended) The method of claim 10, wherein [said] the cell surface molecule is V-CAM-1, I-CAM-1, N-CAM, or V-LAM.

REMARKS

Please amend the application as indicated prior to consideration of this application on the merits. Applicants have filed this continuation application to continue prosecution of claims in the parent application U.S. Serial No. 09/107,051, (the "parent application"). Please amend claims 1 and 7 to 11 and cancel claims 12-20. Applicants reserve the right to pursue the subject matter of the cancelled claims[1] in a separate application.

The specification is merely amended to include a specific reference to the prior application. Claim 1 is amended simply to further define the invention. The amendment of claim 1 is supported by the specification at, for example, page 23, lines 13-16. Claims 7-11 are amended for the sake of consistency.

No new matter would be added by the proposed amendment.

The Invention

Applicants have discovered that bone marrow stromal cells (BMSCs) that have been transfected with an exogenous gene and then cryopreserved express the exogenous gene at a level that is comparable to that seen prior to cryopreservation. This method is now claimed in pending claims 1-11.

35 U.S.C. § 103(a)

Claims 1-5, 7-9, 12-14, 16-18, and 20 were rejected in the parent application as being unpatentable over three references: Greenberger *et al.* (EP 0 381 490; herein, "Greenberger"), Boswell *et al.* (*Exp. Hematol.* 11:315-323, 1983; herein, "Boswell"), and Motta *et al.* (*Bone Marrow Transp.* 12:177, 1993; herein, "Motta").

Applicants have cancelled claims 12-20. Thus, the rejection of claims 12-14, 16-18, and 20 is now moot. In addition, Applicants have amended claim 1. In view of this amendment and the remarks that follow, this ground for rejection should not be repeated in this continuation application.

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[1] These claims covered methods in which bone marrow stromal cells are cryopreserved at a different point in the procedure (*e.g.*, before transfection or before being cultured) than the point claimed in claims 1-11.

In the parent application the the Examiner described the three references cited against the application as follows. Greenberger is characterized as disclosing "a method of gene therapy using genetically modified bone marrow stromal cells" (Final Office Action in parent application at page 2); Boswell is characterized as disclosing that "stromal progenitor cells [are] capable of transferring the hematopoietic microenvironment to liquid culture" (Office Action at page 3); and Motta is characterized as disclosing the successful use of cryopreserved bone marrow cells "after ten years of cryopreservation" (Office Action at page 3).

The Examiner then concluded that "it would have been obvious ... to modify the methods of Greenberger by incorporating cryopreservation of the cultured stromal cells as taught by Boswell or Motta" (Office Action at page 3). The Examiner stated that the required motivation would come from a desire to "avoid steps, time and labor to make the cells again" (Office Action at page 3), and that the required expectation of success would come from "the results of Boswell and Motta," who allegedly showed that "cryopreservation and thawing of marrow would still reproduce the bone marrow microenvironment *in vitro*" (Office Action at page 3).

There is No Motivation to Combine Greenberger with Boswell and Motta

Obviousness can only be established where there is some teaching, suggestion, or motivation to combine or modify the teachings of the prior art. The requisite motivation can be found either in the references themselves or in the knowledge generally available to one of ordinary skill in the art. MPEP at 2143.01.

As noted above, the Examiner suggested in the parent application that one of ordinary skill in the art would have modified the methods of Greenberger by incorporating cryopreservation to "avoid steps, time and labor to make the cells again" (Office Action at page 3). However, the Office Action provided no evidence or reasoning to support this statement. More importantly, nothing in these references describes or suggests cryopreservation of transfected BMSCs. Boswell and Motta both cryopreserve bone marrow, which is a complex mixture of cells containing very few BMSCs (BMSCs constitute only about 0.1% of bone marrow). Nothing in Boswell or Motta suggests cryopreservation of transfected BMSCs. Moreover, nothing in the prior art or in the knowledge generally available to those of ordinary skill in the art suggests that cryopreservation should be carried out *following* BMSC transfection,

as required by the present claims. One cannot arrive at the present invention by incorporating cryopreservation at just any point in a transfection procedure, and there is certainly no suggestion to incorporate it, as applicants have, following BMSC transfection. On this basis alone, the rejection for obviousness should not be applied to amended claim 1.

Greenberger, Boswell, and Motta do Not Suggest all of the Present Claim Limitations

To establish *prima facie* obviousness of a claimed invention, all the claim limitations must be taught or suggested by the prior art. MPEP at 2143.03.

Even if combined, Greenberger, Boswell, and Motta cannot render obvious the method of claim 1 because nothing in these references suggests preparation of BMSCs that express comparable levels of an exogenous gene before and after cryopreservation. As the Examiner admitted in the parent application, "Greenberger et al. do not teach cryopreservation of stromal cells" (Office Action at page 2), and neither Boswell nor Motta preserved transfected cells. Thus, none of the prior art can suggest a method of obtaining a preparation of BMSCs that requires comparable exogenous gene expression before and after cryopreservation. Given this failure, claim 1 should not be rejected.

Greenberger, Boswell, and Motta Fail to Provide the Requisite Expectation of Success

Another requirement for a *prima facie* case of obviousness is that there must be a reasonable expectation of success. MPEP at 2143. The courts have long held that the prior art must not only suggest that something may be tried, but also that the attempt would have a reasonable likelihood of success. *In re Dow Chemical Co.,* 837 F.2d 469, 473 (Fed. Cir. 1988). It is not enough that the prior art render an invention obvious to try. *See, e.g., Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 803 F.2d 1367 (Fed. Cir. 1986) (the district court erred in invalidating the patent on the ground that it was "obvious-to-try").

In the parent case, the Examiner argued that a reasonable expectation of success would come from "the results of Boswell and Motta," which allegedly showed that "cryopreservation and thawing of marrow would still reproduce the bone marrow microenvironment *in vitro*" (Office Action at page 3).

While the Examiner is correct in noting that Boswell and Motta worked with cryopreserved marrow, neither can provide the requisite expectation for success for the method presently claimed. Neither Boswell nor Motta worked with transfected cells, much less transfected BMSC. Thus, neither Boswell nor Motta can shed any light on whether or to what extent cryopreserved BMSCs would express an exogenous gene, let alone whether they would do so to an extent comparable to that seen in BMSCs that have not been cryopreserved (see amended claim 1). Indeed, Motta's relevance to Applicants' claim 1 is quite limited. Motta reports a successful case of hemopoietic reconstitution in a patient (*i.e.*, n = 1) who received a complex mixture of autologous bone marrow cells that were not transfected (as required by the present method). Those of ordinary skill in the art would not have read Motta's anecdotal report as a predictor of success with Applicants' method, particularly given that Motta failed to use (indeed, did not mention) transfected cells. There would have been no way to predict, given Motta's distinct population of non-transfected cells, that applicants' transfected BMSCs could be cryopreserved so that they express an exogenous gene at a level comparable to that in non-cryopreserved cells. Motta's experiments support a single conclusion -- that a complex mixture of non-transfected bone marrow cells can be transplanted despite a long storage period -- and have no predictive value whatsoever for applicants' claimed method.

Boswell similarly fails to disclose BMSCs that have been transfected. Moreover, in attempting to design a new assay for the recovery of stromal cells, Boswell found that even "normal" stromal cells suffered when cryopreserved. For example, Boswell reports that (page 318; emphasis added):

> [w]hen an empiric system of stromal grading was applied to the ... appearance of the stromal lawn ... it was apparent that the frozen group was retarded in extent of stromal development.

Although Boswell's cryopreserved cells later appeared to be comparable to control (*i.e.*, to cells that had not been cryopreserved), it took the cryopreserved cells seven weeks in culture to catch up (see Figure 2). If cryopreserved cells were "retarded" in their ability to establish a stromal layer, one can only expect that cryopreserved cells that are also transfected, as required by applicants' claim 1, would be retarded to at least the same extent (and perhaps more, given that transfection necessarily disrupts the cells' plasma membranes). Indeed, given Boswell's

observation, what one would reasonably expect is that cryopreserved cells that were "retarded" in their ability to establish a stromal layer, would also be retarded in other ways, *e.g.,* in their ability to express an exogenous gene. Certainly, nothing in Boswell would have provided a reasonable basis to expect that transfected BMSCs could be successfully cryopreserved, much less that those cells would express an exogenous gene at levels comparable to those seen in cells that have not been cryopreserved. Accordingly, this ground for rejection should not be repeated in the present application.

Dependent Claim Rejections

Claims 10 and 11 were rejected in the parent case under 35 U.S.C. § 103(a) as being allegedly unpatentable over Greenberger, Boswell, and Motta and further in view of Lobb *et al.* (*Biochem. Biophysic. Res. Comm.* 178:1598-1504, 1991; herein, "Lobb"). The Examiner characterized Lobb as disclosing the expression of a vascular cell adhesion molecule (VCAM1). The Examiner stated that Lobb discloses "that VCAM1 selectively binds to CD8+ memory T cells and should prove useful for immune responses *in vivo*" (Office Action at page 4). This ground for rejection should not be repeated here given the amendment of claim 1, from which claims 10 and 11 depend or ultimately depend.

For the reasons described above, claim 1 would not have been obvious in view of Greenberger, Boswell, and Motta. Since claims 10 and 11 incorporate all of the limitations of claim 1, and since Lobb does nothing to suggest those limitations, claims 10 and 11 cannot be obvious. This ground for rejection should therefore not be repeated here.

Claims 6, 15, and 19 were rejected in the parent application under 35 U.S.C. § 103(a) as being allegedly unpatentable over Lozier *et al.* (*Human Gene Therapy* 5:313-322, 1994; herein, "Lozier") in view of Boswell and Motta. In the parent application the Examiner characterized Lozier as disclosing the preparation of BMSCs from canines with hemophilia B and the transfection of these cells with canine Factor IX (Office Action at page 5).

Claims 15 and 19 have been cancelled. Thus, the rejection of these claims is now moot. With respect to claim 6, the rejection should not be repeated here given the amendment of claim 1, from which claim 6 depends.

For the reasons described above, claim 6 would not have been obvious in view of Greenberger, Boswell, and Motta. Lozier, which focuses on canine cells, can do no more than Greenberger when combined with Boswell and Motta. Moreover, since claim 6 incorporates all of the limitations of claim 1, and since Lozier does nothing to suggest those limitations, claim 6 cannot be obvious. This ground for rejection should not be repeated here.

CONCLUSION

Applicants submit that all of the claims are now in condition for allowance, which action is requested. No fees are believed due in connection with this response. If there are any fees, or any credits, please apply them to Deposit Account No. 06-1050, referencing Attorney Docket No. 07787-004003.

Respectfully submitted,

Date: August 4, 2000

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Attorney's Docket No.: 07787-004003

APPLICATION

FOR

UNITED STATES DESIGN PATENT

TITLE: METHODS OF PREPARING BONE MARROW STROMAL CELLS FOR USE IN GENE THERAPY

APPLICANT: JOEL S. GREENBERGER AND DAVID R. HURWITZ

CERTIFICATE OF MAILING BY EXPRESS MAIL

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August 4, 2000
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Matthew J. Morrissey
Signature

Matthew Morrissey
Typed or Printed Name of Person Signing Certificate

APPLICATION

FOR

UNITED STATES LETTERS PATENT

TITLE: METHODS OF PREPARING BONE MARROW STROMAL CELLS FOR USE IN GENE THERAPY

APPLICANTS: JOEL S. GREENBERGER AND DAVID R. HURWITZ

"EXPRESS MAIL" Mailing Label Number TB426553061US

Date of Deposit DECEMBER 29 1995

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James Gallita

James Gallita

PATENT

ATTORNEY DOCKET NO: 07787/004001

METHODS OF PREPARING BONE MARROW STROMAL CELLS FOR USE IN GENE THERAPY

Background of the Invention

This invention relates to methods of preparing bone marrow stromal cells for use in *ex vivo* gene therapy.

Bone marrow is a complex and dynamic organ system comprised of hematopoietic cells, bone marrow stromal cells, and extracellular matrix. Pluripotent stem cells within the bone marrow proliferate and differentiate into numerous cell types including erythrocytes and leukocytes. It has been known for some time that association between stem cells and stromal cells is critical for this process. Studies in cell culture have shown that a layer of adherent stromal cells must be established before hematopoietic stem cells can grow and differentiate.

Bone marrow stromal cells are a heterogeneous population of cells that are defined by their morphology and function. In cell culture, they have a characteristic, spindle-shaped morphology and secrete growth factors and components that form an extracellular matrix. Stromal cells have been shown to divide in culture in response to epidermal growth factor (EGF; Kimura et al., 1988, Br. J. Hematol. 69:9-12), platelet derived growth factor (PDGF; Kimura et al., *supra*), and basic fibroblast growth factor (bFGF; Kimura et al., *supra*; Oliver et al., 1990, Growth Factors, 3:231-236).

Bone marrow and other biological substances can be frozen according to precalculated time and temperature curves (see, e.g., U.S. Pat. Nos. 4,107,937 and 4,117,881). Cryopreserved materials can be stored for extended periods of time with little degradation (Motta, M.R., (1993), Bone Marrow Trans. 12(2):177). Further, U.S. Pat. No. 4,963,489

indicates that when a suspension of fresh bone marrow and blood is mixed with cryoprotectants and frozen using computerized cryotechnological equipment, 90% of the cells remain viable when thawed. See also, Boswell et al., 1983, Exp. Hematol. 11:315-323; Gilabert et al., 1994, Eur. J. Haematol. 53:93-99; and Rowley, 1992, J. Hematother. Fall:1(3):233-250.

Bone marrow transplantation is a promising therapy for a number of diseases that involve hematopoietic cells. Transplantation can serve to replace cells that have been damaged by an intrinsic disease, such as an anemia, or in instances where hematopoietic cells have been destroyed by chemotherapy or radiation therapy. Transplantation can be autologous, i.e., the patient can serve as his or her own donor. Alternatively, a patient could receive bone marrow from a histocompatible donor. To date, however, conditions for preserving bone marrow, particularly bone marrow stromal cells, which could be used in numerous gene therapies, have not been optimized.

A major obstacle to gene therapies based on the modification of stromal cells is the procurement and sustained availability of therapeutically useful numbers of stromal cells. Consequently, despite the success of bone marrow transplantation, gene therapies that require successful transplantation of bone marrow stromal cells have not yet been realized.

Summary of the Invention

This invention relates to sequential methods of expanding and cryopreserving bone marrow stromal cells that are transfected and used for gene therapy. The invention is based on the discovery that bone marrow stromal cells can be cryopreserved and then transfected, or transfected and then

cryopreserved, and yet still maintain the ability to effectively secrete a desired polypeptide. The cells are plated and expanded according to a particular regimen in complete bone marrow stromal cell medium. With these methods, populations of bone marrow stromal cells can be acquired that are large enough to be useful in a number of therapies. Further, these large populations can be stored for extended periods of time for immediate use when needed.

In general, the invention features a method of preparing bone marrow stromal cells for implantation for gene therapy. The method includes the steps of: (a) obtaining bone marrow stromal cells; (b) culturing the stromal cells to obtain an expanded number of cultured stromal cells; (c) transfecting cultured stromal cells with an exogenous gene to obtain transfected stromal cells; and (d) cryopreserving the transfected stromal cells until implantation.

In another aspect, the method includes the steps of: (a) obtaining bone marrow stromal cells; (b) culturing the stromal cells to obtain an expanded number of cultured stromal cells; (c) cryopreserving the cultured stromal cells; (d) thawing the cryopreserved stromal cells; and (e) transfecting the thawed stromal cells with an exogenous gene prior to implantation.

In another aspect, the method includes the steps of: (a) obtaining bone marrow cells, e.g., from a primary aspirate of bone marrow; (b) cryopreserving the bone marrow cells; (c) thawing the cryopreserved bone marrow cells; (d) culturing the thawed bone marrow cells to obtain an expanded number of cultured stromal cells; and (e) transfecting the cultured stromal cells with an exogenous gene prior to implantation.

In each of these methods, the bone marrow stromal cells can be obtained from bone marrow, e.g., by a primary bone marrow aspirate, a core sample, or by scrapings from one or more bones, from a vertebrate, living or not, e.g., a primate such as a baboon or a human, or mammals in general including dogs, pigs, and cows, or other animals, or can be obtained from bones removed from a vertebrate.

The exogenous gene preferably encodes a secreted peptide such as a serum protein, a blood clotting factor, e.g., factor VIII or factor IX, a cytokine, a lymphokine, a growth factor, e.g., human growth hormone, a peptide hormone, a lipid binding protein, a metabolic enzyme, an antibacterial peptide, an antimicrobial peptide, an antifungal peptide, or a neurotransmitter. The exogneous gene can also encode a cell surface molecule, e.g., V-CAM-1, I-CAM-1, N-CAM, or V-LAM.

In these methods, the culturing or expanding steps can include the steps of: introducing the stromal cells into a vessel pre-coated on an inner surface with a gelatin, e.g., 1.0 percent gelatin in water, and containing a culture medium including an acidic fibroblast growth factor ("aFGF") polypeptide; and expanding the stromal cells in the culture medium under conditions and for a time sufficient to obtain an increased number of bone marrow stromal cells. In these culturing methods, the culture medium further preferably includes at least 0.05 units/ml of a heparin polypeptide. The inner surface of the vessel additionally can be precoated with fetal bovine serum prior to introducing the bone marrow stromal cells.

In particular, the culture medium used in these methods can include 1.0 to 50.0 percent by volume fetal bovine serum, 0.01 to 100.0 ng/ml aFGF polypeptide, and 0.05 to 100 units/ml heparin polypeptide. In a specific

embodiment, the culture medium includes 16.0 percent by volume fetal bovine serum, 1.0 ng/ml aFGF polypeptide, and 5.0 units/ml heparin polypeptide.

The expansion step of the culturing method preferably includes the steps of: (i) removing culture medium and non-adherent cells from the vessel; (ii) adding an amount of fresh culture medium to the vessel; (iii) removing culture medium and non-adherent cells from the vessel and centrifuging the medium and non-adherent cells to form a pellet of non-adherent cells; (iv) resuspending the pellet of non-adherent cells in an amount of culture medium taken from the vessel to form a non-adherent cell mixture; and (v) returning the non-adherent cell mixture to the vessel.

As used herein, an "aFGF polypeptide" is any polypeptide that has an amino acid sequence that is the same as, or substantially identical to, all or a portion of the naturally occurring aFGF protein and which has substantially the same function as the natural or full-length recombinant aFGF as described herein with respect to bone marrow stromal cells. Thus, the term includes recombinant aFGF (e.g., as manufactured by Life Technologies, Inc., Grand Island, N.Y.; #13241-013), "aFGF analogs," i.e., mutant forms of aFGF, and natural or synthetic polypeptide fragments of the full-length aFGF protein and analogs, as long as these analogs and fragments have substantially the same function as natural or full-length recombinant aFGF with respect to bone marrow stromal cells as described herein. These analogs and fragments can easily be tested for their function by using the culture methods described below. Acidic FGF analogs and fragments that do not provide at least 10^7 cells with these methods are not within the present invention.

Similarly, a "heparin polypeptide" is any polypeptide that has an amino acid sequence that is the same as, or substantially identical to, all or a portion of the naturally occurring heparin protein and which has substantially the same function as the natural heparin as described herein with respect to bone marrow stromal cells. Thus, the term includes natural heparin or chemically modified natural heparin, e.g., sodium heparin (ElkinsSinn, Inc., Cherry Hill, NJ), recombinant heparin, "heparin analogs," i.e., mutant forms of heparin, and natural or synthetic polypeptide fragments of the full-length heparin protein and analogs, as long as these analogs and fragments have substantially the same function as natural heparin with respect to bone marrow stromal cells as described herein. The heparin function can be assayed using the culture methods described below.

By "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification, e.g., glycosylation, phosphorylation, or chemical modification, and thus includes natural and synthetic peptides and proteins.

By "mutant form" of aFGF or heparin is meant a polypeptide that includes any change in the amino acid sequence compared to the naturally occurring protein, as long as the mutant form has substantially the same function as the natural or full-length recombinant protein as described herein with respect to bone marrow stromal cells. These changes can arise, e.g., spontaneously by chemical energy, e.g., X-ray, or by other forms of mutagenesis, by genetic engineering, or as a result of mating or other forms of exchange of genetic information encoding the aFGF polypeptide. Mutations can include, for example, substitutions, deletions, insertions, inversions,

translocations, or duplications. The mutations are preferably conservative substitutions, e.g., substitutions within the following groups: glycine alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

The term "identical" as used herein in reference to polypeptides, refers to the amino acid sequence similarity between two polypeptides. When an amino acid position in both of the polypeptides is occupied by identical amino acids, then they are identical at that position. Thus, by "substantially identical" is meant an amino acid sequence that is at least 80%, preferably 85%, more preferably 90%, and most preferably 95% identical to a reference amino acid sequence, and which retains the same functional activity as the reference sequence. Identity of amino acid sequences is typically measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705).

As used herein, "expansion" or "expanding" of cells means culturing cells for a time and under conditions that allow the cells not only to grow and thrive, but to multiply to obtain a greater number of cells at the end of the expansion than at the beginning of the expansion.

As used herein, a "passage" is the process whereby cells that have reached a given number, or a given density, up to and including or beyond confluence, are detached from the tissue culture vessel, collected in an aggregate, such as a pellet formed by centrifugation, and resuspended in tissue culture medium. The suspension is then distributed to tissue culture vessels, such as plates or flasks, in such a way as to provide the cells with a greater total surface

area on which to grow and divide than they had access to previously. This may be done by increasing the number of vessels. For example, the cells growing in one vessel may be detached, collected, resuspended, and distributed to two or more vessels. This process also includes providing the cells with a volume of tissue culture medium that is able to support cellular growth and division.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Fig. 1 is a graph depicting the normalized plasma levels in dogs of human growth hormone (hGH) expressed by genetically modified canine bone marrow stromal cells. The cells transplanted into dogs ALG-3, -9, -10, -11, and -15 were not cryopreserved at any stage of preparation. The cells transplanted into dogs ALG-2 and ALG-4 were cryopreserved before they were transfected. Plasma levels of hGH for each data point were determined in quadruplicate.

Fig. 2 is a bar graph depicting the expression of hGH *in vitro* from transfected canine bone marrow stromal cells. These cultures were established from cells from a sample that was expanded, transfected, cryopreserved, and then thawed. The majority of the cells in the sample were autologously transplanted.

Fig. 3 is a graph that shows the presence of hGH inhibitors in dogs. The inhibitors are shown by comparing the level of hGH assayed by RIA with the amount of hGH "spiked" into the plasma of a dog (ALG-2), over time, following intravenous administration of transfected hGH-expressing stromal cells. Plasma samples were obtained at the times indicated, beginning at day 0. This time point reflects hGH levels in dog plasma "spiked" with 0.5 ng/ml of hGH prior to infusion of the transduced stromal cells. Each sample was assayed in quadruplicate.

Fig. 4 is a graph showing the normalized plasma level of hGH over time in dogs, following autologous transplantation of bone marrow stromal cells. The cells infused into dogs ALG-3, -9, -10, -11, and -15 were not frozen at any stage of preparation. The cells infused into dogs ALG-2 and ALG-4 were expanded, transfected, and then cryopreserved before they were thawed and transplanted. The cells infused into dog ALG-8 were expanded, cryopreserved, thawed, recultured *in vitro*, and then transfected before implantation. Plasma levels of hGH for each data point were determined in quadruplicate.

Fig. 5 is a bar graph that shows the level of hGH in samples of stromal cell-conditioned medium *in vitro*. These cultures were established from cells from a sample that were expanded, cryopreserved, thawed, recultured *in vitro*, and then transfected. The majority of the cells in the sample were autologously transplanted.

Detailed Description

There now follows a description of methods for obtaining bone marrow stromal cells that are suitable for use in transplantation and gene therapy. These methods include the following steps in various orders: obtaining the cells, expanding the cells in culture, transfecting the cells, and cryopreserving the cells. These examples are provided for the purpose of illustrating the invention and should not be construed as limiting.

Expansion, Transfection, and Cryopreservation of Bone Marrow Stromal Cells

Mongrel dogs were fully anesthetized and whole bone marrow was aspirated aseptically from the iliac crest. The aspirate syringes contained heparin to prevent clotting. The bone marrow was transferred from the syringe to a 50 ml conical tube containing 15 mls of a chilled tissue culture medium, such as RPMI or DMEM, and anti-fungal and antibiotic agents (50 μg/ml fungizone; 50 μg/ml gentamicin; 100 units/ml penicillin; 100 μg/ml steptomycin sulfate). Approximately 10-15 mls of bone marrow aspirate was added to each tube and the mixture was kept on ice. Human bone marrow, e.g., from femoral heads, can be obtained by the same techniques, or by other standard techniques.

Nucleated cells were prepared from the bone marrow samples by a standard Ficoll cushion technique. Briefly, 15 ml of FICOLL-PAQUE™ (Pharmacia Biotech) was placed in a 50 ml conical tube and one half of each of the marrow-medium samples was carefully layered on top of the Ficoll. The samples were centrifuged at 400 x g for 30 minutes at 18°C with the brake off so that the centrifuge head decelerated slowly after the elapsed time. The top layer of the resultant preparation, which contained cell-free medium, was

removed and discarded. The middle layer, which contained nucleated cells, was carefully collected and placed into a fresh 50 ml tube containing 20 ml of tissue culture medium, as described above. Additional medium was added to bring the final volume to 50 ml.

The nucleated cells include the bone marrow stromal cells. However, the stromal cells represent only a small fraction, i.e., one in a thousand, of the total number of nucleated bone marrow cells obtained in a bone marrow aspirate.

Expansion

The nucleated cells were collected in a pellet by centrifugation at 100 x g for 10 minutes. The cell pellet was washed with tissue culture medium (RPMI or DMEM with fungizone (25 μg/ml), gentamicin (25 μg/ml), penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml)) and resuspended in 5-10 ml of "complete bone marrow stromal cell medium" ("complete medium"). After resuspension the cells were counted.

Generally, the complete bone marrow stromal medium contains the following ingredients in the following ranges of amounts or concentrations. DMEM with 1 to 50% fetal bovine serum (FBS)(preferably greater than 12.5%) by volume; 0.01 to 100 ng/ml of an aFGF polypeptide, e.g., a recombinant aFGF; 0.05 to 100 units/ml of a heparin polypeptide, e.g., sodium heparin; 0.25 to 250 μg/ml of fungizone; 0.25 to 250 μg/ml of gentamicin; 1 to 1000 units/ml penicillin; and 1 to 1000 μg/ml of streptomycin sulfate. As used in the experiments described below, the complete medium contained DMEM with 16 percent by volume heat-inactivated FBS, recombinant aFGF (1 ng/ml) by volume, heparin (5 units/ml), fungizone (25 μg/ml), gentamicin (25

μg/ml), penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml).

Tissue culture flasks (T150 cm^2) are preferably coated initially with gelatin and FBS. Specifically, a solution of gelatin (Sigma; 1% in water) was added to each flask until the bottom of the flask was just covered. The excess was removed and the flasks were left undisturbed, bottom side down, at room temperature, for at least 30 minutes. The flasks can be refrigerated at this point for later use. Heat-inactivated FBS was then added to the gelatinized flasks. As before, the excess solution was removed and the flasks were left, bottom side down, at room temperature for at least 30 minutes. The flasks were either used at this point or refrigerated.

The nucleated cells of the bone marrow, prepared as described above, were added to the coated flasks at approximately 1 X 10^8 cells/T150 flask. The cells were incubated in 15 mls of complete bone marrow medium, at 33°C, in the presence of 5% CO_2. After 3-4 days, or when the stromal cells have adhered to the inner surface of the tissue culture vessel, 15 ml of complete medium was added to the cultures, dropwise, so the cells were not disturbed. One week later, before vital components within the medium are depleted, the so-called "conditioned medium," i.e., the medium in the flask which contains non-adherent cells, was removed, and 15 ml of fresh complete medium was added to the flask. The non-adherent cells were pelleted by centrifugation at 500 x g for 5 minutes, resuspended in 15 ml of conditioned medium and returned to the original flask. Thus, the non-adherent cells were returned to the flask and the medium was changed in such a way that it contained one part fresh medium and one part conditioned medium.

In general, the key to this regimen of cell culture is to: (1) coat the inner surface of the tissue culture vessel with a solution of gelatin, (2) keep returning the non-adherent cells to the culture when exchanging the medium, (3) add medium that contains sufficient nutrients to sustain growth without removing all of the substances secreted by the bone marrow cells, which enhance their growth, (4) supplement the tissue culture medium with aFGF, and (5) supplement the tissue culture medium with heparin.

This process, where the non-adherent cells are removed, pelleted, and returned to the culture with equal parts of fresh and conditioned medium, is repeated once a week, for 2-3 weeks or until a monolayer of adherent cells has formed. Once the monolayer of bone marrow stromal cells developed, the cells were passaged by splitting them 1:2 or 1:3 into fresh flasks. At this point, and from this point on, the flasks were coated with gelatin but not with FBS. It is also no longer necessary to feed the established stromal cells with conditioned medium or to return non-adherent cells to the culture. The cells may be passaged in this manner at least 8 times or more.

This method can be used to select and expand canine or human (or other vertebrate) bone marrow stromal cells, to develop a total cell number of more than 10^8, and even more than 3×10^9 *in vitro*, from bone aspirates of individual subjects. Other techniques of obtaining bone marrow can be used. The bone marrow stromal cells obtained from dogs by this method exhibited the characteristic appearance of fibroblast-like bone marrow stromal cells. Given that the success of gene therapy depends on the cellular production of adequate levels of the transgene product, which can be quite low, the ability to expand cells in culture to 10^8 to 10^9 or more represents a substantial improvement.

Transfection

To determine whether bone marrow stomal cells that were grown according to the methods described above could be transfected, the plasmid expression vector pETKhGH was prepared and transfected into canine stromal cells using standard techniques. The dog model is an accepted animal model of the human bone marrow system, and results in dog studies are reasonably predictive of efficacy in human patients.

The vector was prepared from pTKGH (Selden et al., 1986, Mol. Cell. Biol., 6:3173-3179), which is comprised of the human growth hormone (hGH) gene, including introns, under the transcriptional regulation of HSV thymidine kinase (TK) promoter sequences (Nichols Institute Diagnostics, San Juan Capistrano, CA). In addition, a 179 base pair FokI-PvuII restriction enzyme fragment from the SV40 enhancer (as described in Hurwitz et al., 1987, Nucl. Acids Res. 15:7137-7153) was tailed with HindIII sites by PCR using a derivative of the pSV(E)-MLP plasmid (Hurwitz et al., *supra*) as a template, and cloned into the HindIII site of pTKGH just upstream of the TK promoter. The pETKhGH plasmid lacks a eukaryotic origin of replication and does not integrate into the host cell genome. As such, the vector expresses hGH transiently.

By simply varying which gene is incorporated, vectors can be made that would express secreted proteins such as serum proteins, clotting factors, e.g., factor VIII and factor IX, cytokines, lymphokines, growth factors, e.g., human growth hormone, peptide hormones, lipid binding proteins, metabolic enzymes, antibacterial agents, antimicrobial agents, antifungal agents or neurotransmitters. Similarly, the vector could express cell surface components, e.g. receptors or cell surface adhesion

molecules such as vascular cell adhesion molecule-1 (V-CAM1), intercellular adhesion molecule-1 (I-CAM-1), or V-LAM, or secreted gene products, such as collagen.

Methods of engineering plasmid expression vectors are well known to persons of ordinary skill in the art, and many expression vectors that contain genes encoding proteins within the families listed above have been constructed and can be used to transfect bone marrow stromal cells. These include viral vectors that encode cytokines, such as IL-6 (Whartenby, et al., 1995, Pharmacology and Therapeutics, 66:175-190), and IL-7 (Kim et al., 1994, Human Gene Ther., 5: 1457-1466); clotting factors such as factor VIII (Dwarki et al., 1995, Proc. Natl. Acad. Sci., USA, 92:1023-1027), and factor IX (Palmer, et al., 1989; Blood, 73:438-445); metabolic enzymes, such as aspartyl-glucosaminidase (Enomaa et al., 1995, Human Gene Ther., 6:723-731), purine nucleoside phosphorylase (Jorisson et al., 1995, Human Gene Ther., 6:611-623), and uroporphyrinogen III synthase (Moreau-Gaudry et al., 1995, Human Gene Ther., 6:13-20); as well as cell surface adhesion molecules such as I-CAM-1 (Pilewski et al., 1995, AM. J. Resp. Cell. Mol. Biol. 12:142-148). In addition, useful plasmid expression vectors have been constructed for the expression of V-CAM-1 (Lobb et al., 1991, Biochem. Biophys. Res. Comm., 78:1498-1504), and neural cell adhesion molecule (N-CAM; Woo et al., 1993, Exp. Cell Res. 204:336-345).

In the present example, canine bone marrow stromal cells were transfected with pETKhGH by either the $CaPO_4$-DNA coprecipitation method, using the MBS Mammalian Transfection Kit (Stratagene Cloning Systems, LaJolla, CA), or the cationic lipid-DNA complex method using LIPOFECTAMINE® reagent and OPTI-MEM® I reduced-serum medium (Life Technologies) according to the manufacturer's instruction.

Several batches of expanded stromal cells from a dog designated ALG-2 were transfected with the calcium-phosphate method and one batch of cells from dog ALG-4 was transfected by lipofection. The amount of hGH secreted into the complete medium was measured by a radioimmunoassay during a 24 hour period. These data are shown in Table 1. Cells from dog ALG-2 secreted a total of 1,818 μg of hGH and cells from dog ALG-4 secreted 824 μg of hGH during this time.

In column 7 of Table 1, the hGH expression is based on *in vitro* expression during the 24 hour period prior to cryopreservation. All cells derived from dog ALG-2 (data in rows 1-5 of Table 1) were transfected with the MBS Mammalian Transfection Kit from Stratagene or ProFection Mammalian Transfection Systems from Promega. The data in the last row, from dog ALG-4-derived cells were transfected with the LIPOFECTAMINE reagent from Life Technologies, Inc.

Table 1: Ex Vivo Transfection of Bone Marrow Stromal Cells with the pETKhGH Plasmid Expression Vector Prior to Cryopreservation

ALG Dog# (USDA #)	Method of Transfection (Date of Transfection)	Passage # of Transfected Cells	# of Cells Seeded for Transfection /T150 Flask	# of T1150 Flasks Transfected (& Survive)	# of Cells Recovered and Frozen	hGH Expression (ug/10^6 cells/ 24 hr)	Total hGH Expression Capacity (ug/24 hr)
#2 (82716)	Calcium Phosphate (6/18 or 7/6 or 7/10/94)	P11 or P12	A Collection of 100 mm Dishes and T150 Flasks	A Collection of 100 mm Dishes and T150 Flasks	8.81×10^7	0.294	25.9
	Calcium Phosphate (8/4/94)	P12	3.3×10^6	48	6.3×10^8	0.198	125
	Calcium Phosphate (8/11/94)	P13	5×10^6	50	1.98×10^9	0.161	318
	Calcium Phosphate (8/18/94)	P14	5×10^6	18	3.39×10^8	0.313	106
	Calcium Phosphate (8/11/94)	P15	4.9×10^6	42	3.14×10^8	3.11	1243
				TOTAL	3.35×10^9		1818
#4 (89711)	Lipofection (8/25/94)	P3	4×10^6	41	2.85×10^8	2.89	824

Cryopreservation

Immediately after the assessment of hGH production, the expanded stromal cells were frozen. In preparation for cryopreservation, the cells were rinsed once with Dulbecco's Phosphate Buffered Saline (Gibco #14190-144) and detached with Trypsin-EDTA (0.05% Trypsin, 0.53 mM tetra-sodium-EDTA; Gibco #25300-062). The trypsinization was stopped by adding an equal volume of media (DMEM with antibacterial and anti-fungal agents at the concentrations given above). The cells were pelleted by centrifugation at 500 x g for 5 minutes, resuspended in 3 ml of media, and counted. Cell density was adjusted to 1 x 10^6 cells/ml with media containing 10% Dimethyl Sulfoxide (DMSO; Sigma D-8779), and 1 ml aliquots were added to sterile 2 ml cryogenic vials (Corning #25704). The vials were immediately stored at -80°C overnight. After 24 hours, the vials were transferred to a liquid nitrogen tank or to a -150°C freezer for long-term storage. 3.35 x 10^9 cells derived from dog ALG-2 and 2.85 x 10^8 cells derived from dog ALG-4 were cryopreserved in this manner.

For larger scale cryopreservation, after trypsinization, the cells are pelleted by centrifugation at 500 x g in larger volumes of medium, e.g., 200 ml or more. The cell pellet is suspended in 10 to 20 ml of medium, and cells are counted. The suspension is then brought to a larger volume, e.g., 45 ml, with medium, and added to a transfer pack container (Baxter Fenwal, 4R2001) with a sterile syringe fitted with an 18 gauge sterile needle. Five ml of DMSO is then added, and the pack is stored at -80°C overnight. After 24 hours, the pack is transferred to liquid nitrogen tanks or to a -150°C freezer for long-term storage. Both of these cryopreservation methods can be used

for stromal cells from humans as well as from dogs, primates, cows, pigs, and other animals.

Transplantation

Stromal cells that had been modified *ex vivo* and cryopreserved were later thawed, washed, and re-infused into a foreleg vein of the dogs in an autologous manner. Both animals had previously received autologous infusions of cells that had been cultured but that were not modified with an hGH expression vector. The expression capacity of the cells infused into dog ALG-2 was 1,400 μg hGH/24 hr and, for dog ALG-4, 575 μg hGH/24 hr (Table 2). The expression capacities were determined by the number of cells infused and the level of hGH expression *in vitro* during the 24 hour period preceding cryopreservation.

The data shown in Table 2 were derived from cells that were reintroduced intravenously into a foreleg vein. The hGH expression capacities, of 1,400 and 575 μg/24 hours, shown in the last column were based on *in vitro* expression during the 24 hour period prior to cryopreservation. The value of 445 μg/24 hours, again in the last column, was also based on *in vitro* expression. In this instance, an aliquot of cells was taken from the pool of cells that were reinfused and returned to tissue culture. hGH was measured during the 24 hour period immediately after the remainder of the cells were reinfused.

Some cells derived from dog ALG-4 were returned to tissue culture, and hGH was measured *in vitro* for 24 hours after the sibling cells were reinfused. During this time, the cells in culture expressed and secreted 2.24 μg of hGH/1 x 10^6 cells/24 hours, which predicts an *in vivo* expression capacity of 445 μg of hGH/24 hr period (Table 2). The same measurements were taken before cryopreservation. At that time, the cells in culture expressed and secreted

2.89 μg of hGH/1 x 106 cells/24 hours, which predicts an expression capacity *in vivo* of 575 μg/24 hours. Note that the measurements taken before cryopreservation are very similar to those obtained after cryopreservation.

Normalized plasma levels were also similar whether or not the dogs had previously been infused with autologous bone marrow stromal cells. These data indicate that cryopreservation does not significantly alter the ability of transfected stromal cells to express hGH. Furthermore, these data demonstrate that *in vitro* expression levels are reliable indicators of the subsequent level of *in vivo* hGH expression, regardless of whether or not the cells have been cryopreserved. Thus, *in vitro* levels of hGH expression prior to cryopreservation can be used to determine a therapeutically useful number of cells for transplantation.

Secretion of hGH *in vivo*, into peripheral blood plasma, continued for 3 days following reintroduction of the modified stromal cells into both dogs ALG-2 and ALG-4, achieving a peak plasma level of 1.76 ng/ml in dog ALG-2 (Table 3).

In Table 3, the lowest hGH standards, shown in column 2, are statistically higher (t-test) than the expression of hGH in pre-infusion plasma at a confidence level of greater than 95%. The average plasma levels of hGH, shown in column 4, represent values that are statistically higher (t-test) than those seen in pre-infusion plasma at a confidence level of greater than 99%, except where indicated. The data above the thick line in Table 3 pertains to ALG-2, and data below the thick line pertains to ALG-4.

Table 2: Reintroduction into Dogs of Thawed Autologous Bone Marrow Stromal Cells Transfected with the pETKhGH Plasmid Expression Vector Prior to Cryopreservation

ALG Dog#	Date of Reintroduction of Cells	Method of Reintroduction of Cells	Number of Cells	Cell Conc. (cells/ml) & Volume	Previous Autologous Stromal Cell Infusion?	hGH Expression Capacity (ug/24 hr)
#2	9/7/94	Intravenous	2.58×10^9	1.29×10^7 200ml	Yes	1,400
#4	5/16/95	Intravenous	1.99×10^8	1.99×10^6	Yes	575 445

Table 3: Plasma Levels of hGH Expressed In Vivo by Reintroduced Ex Vivo Modified Autologous Bone Marrow Stromal Cells

Days (Hours) Following Reintroduction of Cells	Assay Standard Range	Number of Assayed Replicates of Sample	Average Plasma Level of hGH +/−Assay Standard Error
1 (24 hr)	0.20 −5.0 ng/ml	5	1.76 ng/ml (+/−0.03)
2 (48 hr)	0.05 −5.0 ng/ml	3	0.425 ng/ml (+/−0.013)
3 (72 hr)	0.05 −5.0 ng/ml	3	0.036 ng/ml (+/−0.006)(98%)
4−13	0.05 −5.0 ng/ml	4	Undetectable (i.e., not above background controls at the 95% level)
0 (0.25)	0.011 −1.10 ng/ml	4	0.314 ng/ml (+/−0.009)
1 (18 hr)	0.011 −1.10 ng/ml	4	0.169 ng/ml (+/−0.004)
1 (24 hr)	0.011 −1.10 ng/ml	4	0.174 ng/ml (+/−0.009)
2 (42 hr)	0.011 −1.10 ng/ml	4	0.110 ng/ml (+/−0.009)
2 (48 hr)	0.011 −1.10 ng/ml	4	0.070 ng/ml (+/−0.004)
3 (66 hr)	0.011 −1.10 ng/ml	4	0.021 ng/ml (+/−0.002)
3 (72 hr)	0.011 −1.10 ng/ml	4	0.011 ng/ml (+/−0.001)(98%)
4 −7	0.011 −1.10 ng/ml	4	Undetectable (i.e., not above background controls at the 95% level)

The longest known half-life of hGH is 26 minutes (in normal humans or guinea pigs; Holl et al., 1993, J. Clin. Endocrinol. Metab., 77:216). Assuming that hGH in canine plasma has a similar half-life, any hGH that was present would be reduced to less than 0.01 ng/ml, which is undetectable by radioimmunoassay, in less than three hours. Therefore, the hGH detected in the plasma is the result of *de novo* expression from reintroduced *ex vivo* modified bone marrow stromal cells.

In other studies, plasma levels of hGH from transplanted stromal cells that were genetically modified as described here, but that were not cryopreserved at any stage, were determined. The normalized *in vivo* levels of hGH expression were comparable, whether or not the genetically modified stromal cells were cryopreserved before reinfusion. Levels of hGH in the plasma of each dog, at each time point, were normalized by dividing absolute hGH plasma levels (ng/ml) by the total hGH expression capacity (μg/24 hr) of the cells infused into each dog, multiplied by the weight (kg) of the individual dog, and then by 100%.

As shown in Fig. 1, data collected from dogs ALG-2 and ALG-4 show the plasma level of hGH in the dogs produced by cells that have been cryopreserved after transfection, and data collected from dogs ALG-3, -9, -10, -11, and -15 show the plasma level of hGH secreted by cells that were infused without being frozen. The results vary from dog to dog, but show that the cryopreserved cells and non-cryopreserved cells worked essentially the same. Thus, these data support the use of cryopreservation as a means to repeatedly transplant *ex vivo* expanded bone marrow stromal cells.

Although the majority of the vector-modified cells were used for transplantation, a small number of these cells

were returned to tissue culture. Periodically, samples of tissue culture medium that had been conditioned by these cells were assayed for hGH. The cultured cells, which serve as a model for the reintroduced cells, expressed and secreted significant levels of hGH *in vitro*. Although the level of expression decreased over time, cells were shown to express hGH *in vitro* for as long as 15 weeks after the time sibling cells were reintroduced to animals (Fig. 2). Fig. 2 shows that the level of hGH peaked at 4 or 3 (flasks 1 and 2, respectively) μg/24 hr/10^6 cells, was still over 1 μg/24 hr/10^6 cells, and then dropped gradually until week 15.

Circulating stromal cells bearing the hGH vector were detected by a sensitive nested PCR assay. This assay can detect 5-10 copies of the pETKhGH plasmid expression vector in a background of 1 x 10^5 control stromal cells and is effective in 90% of the reactions performed (9 positives/10 reactions).

In preparation for the nested PCR assay, blood samples were obtained after transfected stromal cells were infused. These samples were subjected to a Ficol gradient and bone marrow stromal cells from the buffy coat fraction were isolated. Cells obtained from the buffy coat of dog ALG-4 prior to the autologous transplantion of stromal cells served as the negative control. Cells obtained from the buffy coat were incubated in cell lysis buffer (1 x 10^7 cells/ml) at 55°C, overnight. Lysis buffer is comprised of 50 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.5% Tween 20 and 200 μg/ml Proteinase K (Boehringer Mannheim Indianapolis, IN). Cell debris was removed by centrifugation in an Eppendorf microcentrifuge for 10 minutes. The lysate supernatant was incubated at 95°C for 10 minutes and subsequently used in PCR experiments.

The nested PCR reaction was performed in two steps. In the first step, 25 μl of cell lysate (2.5 X 10^5 cell equivalents) was mixed with 25 μl of 2X PCR reaction mix and subjected to an initial incubation at 94°C for 5 minutes and then 35 cycles of 94°C (30 seconds), 58°C (50 seconds), and 72°C (1.5 minutes) in the presence of a 5'-oligonucleotide primer that is homologous to exon 2 sequences in the hGH gene, and a 3'-primer that is homologous to exon 5 sequences in the hGH gene. Primers were obtained from Oligos, etc. Inc. (Wilsonville, OR). The final concentration of the PCR reaction mix was 50 mM Tris-HCl (pH 9.0), 50 mM NaCl, 2 mM $MgCl_2$, 0.2 mM each of NTPs (dATP, dGTP, dCTP, and dTTP), 0.5 μM each of 5'- and 3'-primers, and 0.5 units Taq DNA polymerase (Promega Corp., Madison, WI).

For the second nested PCR amplification, 1 μl of the first PCR reaction product was added to 49 μl of 1X PCR reaction mix in the presence of a 5'-primer that is homologous to exon 2 sequences in the hGH gene, and a 3'-primer that is homologous to exon 5 sequences. Importantly, this second set of oligos is homologous to gene sequences internal to the first set of oligos and did not overlap their sequences. All primers were chosen from regions of the template gene that did not contain internally repetitive sequences and that had a G-C content of less than 67%. Numerous other primer combinations can be developed by standard techniques for use in this nested PCR assay.

Thermocycling for the nested reaction was identical to the first reaction except that the annealing temperature was 56°C. A 10 μl aliquot of each of the first and nested PCR reaction products was analyzed by electrophoresis on a 1% agarose gel and the amplified cDNA was visualized by ethidium bromide staining. DNA molecular weight markers were comprised of phiX174 DNA digested with HaeIII

(Boehringer Mannheim). The expected size of the correct nested PCR product is 1,119 base pairs. Control analyses on canine cells demonstrated that the nested PCR reaction protocol did not detect canine growth hormone gene sequences.

Nested PCR analysis revealed that vector-modified stromal cells were present in the peripheral circulation of animals for 15 weeks after cells were reintroduced to the circulation.

Following the reintroduction of hGH-expressing stromal cells, hGH inhibitors developed *in vivo* (Fig. 3; dog ALG-2). These inhibitors were detected by experiments in which plasma samples were spiked with 0.5 ng/ml of hGH at various times. As shown in Fig. 3, the first hGH measurement (indicated on Fig. 3 as day 0) was obtained prior to infusion of transduced cells. These measurements continued for 20 days following infusion of hGH-expressing stromal cells. Inhibitor activity was determined by comparing the level of hGH in each sample, which was empirically measured by RIA, with the amount of hGH (0.5 ng/ml) purposefully added to each sample.

On days 0 to 8, the percentage of hGH detected was between about 75 (0.75) to 90% (0.9), which served as a control level. Inhibitors in the peripheral blood plasma were evident 9 days after modified stromal cells were reintroduced *in vivo* and, by 15 days, their level rose to a point sufficient to prevent detection of 93% of the hGH that had been purposefully added to plasma at 0.5 ng/ml. The presence of these inhibitors provides further evidence that the expression vector is producing biologically active hGH.

Expansion, Cryopreservation, and
Transfection of Bone Marrow Stromal Cells

In certain circumstances, it is desirable to expand bone marrow stromal cells in culture and cryopreserve them prior to transduction. To develop such a procedure, bone marrow stromal cells were aspirated from the primary iliac crest of dog ALG-8, and expanded *in vitro* by culturing methods described in detail above. After the second passage, 2.12 x 10^8 cells were cryopreserved in media containing DMSO by the procedure described in detail above. From this frozen stock, 1 x 10^8 cells were subsequently thawed and placed in tissue culture as passage 3 cells. After one additional passage, 1.35 x 10^8 cells were transduced by transfection with the pETKhGH plasmid expression vector. The level of *in vitro* expression and secretion of hGH into the tissue culture media was 1.22 μg/1 x 10^6 cells/24 hr period, as determined by radioimmunoassay.

These cells (1.11 x 10^8 total cells with an hGH expression capacity of 135 μg/24 hr period) were reintroduced into dog ALG-8 by infusion into a foreleg vein (Table 4). Dog ALG-8 had not previously received autologous bone marrow stromal cells. Secretion of hGH into the peripheral blood plasma was demonstrated for 3 days after the cellular infusion and achieved a peak plasma level of 0.24 ng/ml (Table 5). Given that the half-life of hGH in canine plasma should not be more than 26 minutes, any hGH that was present would fall below the level of sensitivity of the radioimmunoassay (0.01 ng/ml) in less than 1.7 hours in this case. In addition, the levels after infusion (e.g. 0.189 ng/ml at 24 hours; Table 5) would be undetectable in less than 2 hours. Therefore, the hGH present in the plasma

3 days after modified stromal cell infusion must be due to *de novo* expression of hGH by these cells.

The hGH expression capacity shown in Table 4 was based on *in vitro* expression of hGH during the 24 hour period preceeding cryopreservation. In Table 5, the lowest hGH standards, shown in column 2, are statistically higher (t-test) than the expression of hGH in pre-infusion plasma at a confidence level of greater than 95%. The average plasma levels of hGH, shown in column 4, represent values that are statistically higher (t-test) than those seen in pre-infusion plasma at a confidence level of greater than 99%, except where indicated.

Table 4: Reintroduction into ALG of Autologous Bone Marrow Stromal; Cells Transfected with the pETKhGH Plasmid Expression Vector Subsequent to Cryopreservation

ALG Dog# (USDA #)	Date of Reintroduction of Cells	Method of Reintroduction of Cells	Number of Cells	Cell Conc. (cells/ml) & Volume	Previous Autologous Stromal Cell Infusion?	hGH Expression Capacity (ug/24 hr)
#8	(1/23/95)	Intravenous	1.11×10^8	9.65×10^5 115 ml	No	135

Table 5: Plasma Levels of hGH Expressed In Vivo by Reintroduced Ex Vivo Modified Autologous Bone Marrow Stromal Cells (ALG-8)

Days (Hours) Following Reintroduction of Cells	Assay Standard Range	Number of Assayed Replicates of Sample	Average Plasma Level of hGH +/-Assay Standard Error
0 (0.25)	0.04 -1.0 ng/ml	4	0.240 ng/ml (+/-0.005)
1 (18.5 hr)	0.04 -1.0 ng/ml	4	0.194 ng/ml (+/-0.006)
1 (24 hr)	0.04 -1.0 ng/ml	4	0.189 ng/ml (+/-0.005)
2 (42 hr)	0.04 -1.0 ng/ml	4	0.136 ng/ml (+/-0.002)
2 (48 hr)	0.03 -0.10 ng/ml	4	0.092 ng/ml (+/-0.001)
3 (66.5 hr)	0.04 -0.10 ng/ml	4	0.057 ng/ml (+/-0.003)
3 (72 hr)	0.03 -0.10 ng/ml	4	0.051 ng/ml (+/-0.003)
4 -14	0.03 -0.10 ng/ml	4	Undetectable (i.e., not above background controls at the 95% level)

To determine whether cryopreservation prior to transduction affected the ability of the cells to secrete hGH *in vivo*, normalized plasma levels of hGH were measured from bone marrow stromal cells that had never been cryopreserved (Fig. 4, samples from dogs ALG-3, -9, -10, -11, and 15). These levels were compared with those from stromal cells that were expanded *in vitro*, cryopreserved, thawed, and transduced with the hGH expression vector prior to reintroduction into animals (dog ALG-8). The normalized *in vivo* expression levels were comparable, regardless of whether or not the cells had been cryopreserved. Levels of hGH in the plasma of each dog, at each time point, were normalized by dividing absolute hGH plasma levels (ng/ml) by the total hGH expression capacity (μg/24 hr) of the cells infused into each dog, multiplied by the weight (kg) of the individual dog, and then by 100%. Furthermore, hGH expression was comparable regardless of whether the cells were cryopreserved before (dog ALG-8) or after (dogs ALG-2 and ALG-4) they were genetically modified.

To determine whether the cells transducted and infused to dog ALG-8 maintained the ability to express hGH *in vitro*, a small number of the cells were kept in culture while the majority of cells were reintroduced into dog ALG-8. Periodically, samples of conditioned medium were assayed for the expression and secretion of hGH and the cells were counted at the time of passage. The cultured cells expressed and secreted significant levels of hGH. Although levels of expression decreased over time, hGH could be detected *in vitro* for at least 5 weeks after the sibling cells were reintroduced into ALG-8 (Fig. 5).

Plasma levels of hGH were detected in dog ALG-8 as soon as 15 minutes following the infusion of cells. To determine the level of expression of infused cells for this

period, a 1 ml aliquot of the modified cells that were intended for reinfusion to ALG-8 was kept on ice while the rest of the cells were infused. Complete medium (5 ml) was added to the aliquot and the cells were kept at 37°C for 20 minutes. Subsequently the cells were washed, resuspended in tissue culture medium, and the amount of hGH expressed and secreted into the medium was determined at a time that reflected the 15 minute period post-infusion measurement from plasma.

The *in vitro* expression capacity was 5.58 ng/1 x 10^6 cells/15 minutes. Therefore, the 1.11 x 10^8 cells that were infused would express 619 ng of hGH/15 minutes. This in turn, corresponds to a plasma concentration of 0.867 ng/ml (for ALG-8; Table 6). This value is 3.6-fold higher than the assayed level of 0.24 ng/ml of hGH in the plasma fifteen minutes following the end of the infusion of the cells (Table 5). This demonstrated that the infused cells express sufficient hGH in 15 minutes to account for the hGH level seen in plasma 15 minutes after infusion. The level assayed in plasma is probably lower than that predicted by *in vitro* analysis because of distribution within extravascular, as well as intravascular, compartments.

Table 6: In Vitro Expression of hGH During a 15 Minute Time Period from a Sample of ALG Bone Marrow Stromal Cells at Time of Infusion of Sibling Cells

Determined Expression of hGH ($ng/10^6$ cells/15 min)	Estimated Expression Capacity of Infused Cells (1.11×10^8 cells) (ng/15 min)	Estimated Plasma Volume of Dogs	Estimated Plasma Concentration of hGH Due to Expression in 15 min (ng/ml)
5.58 ng	619 ng	714	0.867 ng/ml

Cryopreservation of Primary Bone Marrow
and Subsequent Establishment, Expansion,
and Transfection of Bone Marrow Stromal Cells

Primary bone marrow aspirate was prepared using a Ficoll gradient, as described above. A small aliquot of 1.9×10^7 cells, which represented about 10% of the total, was cryopreserved. In preparation for cryopreservation, cells were suspended in 50% medium, 50% FBS at a density of $2\text{-}5 \times 10^7$ cells/ml. 900 μl of this suspension was aliquoted into 2 ml sterile cryogenic vials (Corning #25704) with 100 μl of DMSO. The vials were stored at -80°C for 24 hours and then transferred to a -150°C freezer or to liquid nitrogen tanks for long-term storage. Human primary bone marrow aspirates can be cryopreserved in the same manner.

Cryopreserved cells were subsequently thawed and bone marrow stromal cells established and expanded in culture as described above. After the fourth passage, two T25 flasks containing 4×10^5 stromal cells each were transfected with the pETKhGH expression vector using LipofectAMINE®. Transduced stromal cells expressed and secreted hGH into the media. Two weeks following transfection, hGH was expressed at 143 ng and 155 ng per 1×10^6 cells per 24 hours in two flasks and expression of hGH continued for 9 weeks.

These results demonstrate that bone marrow stromal cells can be expanded from primary bone marrow aspirates that have been cryopreserved and that these cells can be transduced at a later time and express transgene product.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to

illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. A method of preparing bone marrow stromal cells for implantation for gene therapy, said method comprising:

(a) obtaining bone marrow stromal cells;

(b) culturing the stromal cells to obtain an expanded number of cultured stromal cells;

(c) transfecting cultured stromal cells with an exogenous gene to obtain transfected stromal cells; and

(d) cryopreserving the transfected stromal cells until implantation.

2. The method of claim 1, wherein the bone marrow stromal cells are obtained from bone marrow from a vertebrate.

3. The method of claim 1, wherein the bone marrow stromal cells are obtained from bones removed from a vertebrate.

4. The method of claim 1, wherein the bone marrow stromal cells are mammalian.

5. The method of claim 4, wherein the bone marrow stromal cells are human.

6. The method of claim 4, wherein the bone marrow stromal cells are canine.

7. The method of claim 1, wherein said exogenous gene encodes a secreted peptide.

8. The method of claim 7, wherein said secreted peptide is a serum protein, a blood clotting factor, a cytokine, a lymphokine, a growth factor, a peptide hormone, a lipid binding protein, a metabolic enzyme, an antibacterial peptide, an antimicrobial peptide, an antifungal peptide, or a neurotransmitter.

9. The method of claim 8, wherein said blood clotting factor is factor VIII or factor IX.

10. The method of claim 1, wherein said exogenous gene encodes a cell surface molecule.

11. The method of claim 10, wherein said cell surface molecule is V-CAM-1, I-CAM-1, N-CAM, or V-LAM.

12. A method of preparing bone marrow stromal cells for implantation for gene therapy, said method comprising:

(a) obtaining bone marrow stromal cells;

(b) culturing the stromal cells to obtain an expanded number of cultured stromal cells;

(c) cryopreserving the cultured stromal cells;

(d) thawing the cryopreserved stromal cells; and

(e) transfecting the thawed stromal cells with an exogenous gene prior to implantation.

13. The method of claim 12, wherein the bone marrow stromal cells are mammalian.

14. The method of claim 12, wherein the bone marrow stromal cells are human.

15. The method of claim 12, wherein the bone marrow stromal cells are canine.

16. The method of claim 12, wherein said exogenous gene encodes a secreted peptide.

17. A method of preparing bone marrow stromal cells for implantation for gene therapy, said method comprising:

(a) obtaining bone marrow cells from bone marrow;

(b) cryopreserving the bone marrow cells;

(c) thawing the cryopreserved bone marrow cells;

(d) culturing the thawed bone marrow cells to obtain an expanded number of cultured stromal cells; and

(e) transfecting the cultured stromal cells with an exogenous gene prior to implantation.

18. The method of claim 17, wherein the bone marrow stromal cells are human.

19. The method of claim 17, wherein the bone marrow stromal cells are canine.

20. The method of claim 17, wherein said exogenous gene encodes a secreted peptide.

Abstract of the Disclosure

This invention relates to sequential methods of cryopreserving bone marrow stromal cells that are transfected and used for gene therapy by transplantation. These methods include the following steps in various orders: obtaining the cells, expanding the cells in culture, transfecting the cells, and cryopreserving the cells. With these methods, populations of bone marrow stromal cells can be acquired that are large enough to be useful in a number of therapies. Further, these large populations can be stored for extended periods of time for immediate use when needed.

157449.B11

INFORMAL DRAWINGS FOR APPLICATION

FOR

UNITED STATES DESIGN PATENT

TITLE: METHODS OF PREPARING BONE MARROW STROMAL CELLS FOR USE IN GENE THERAPY

APPLICANT: JOEL S. GREENBERGER AND DAVID R. HURWITZ

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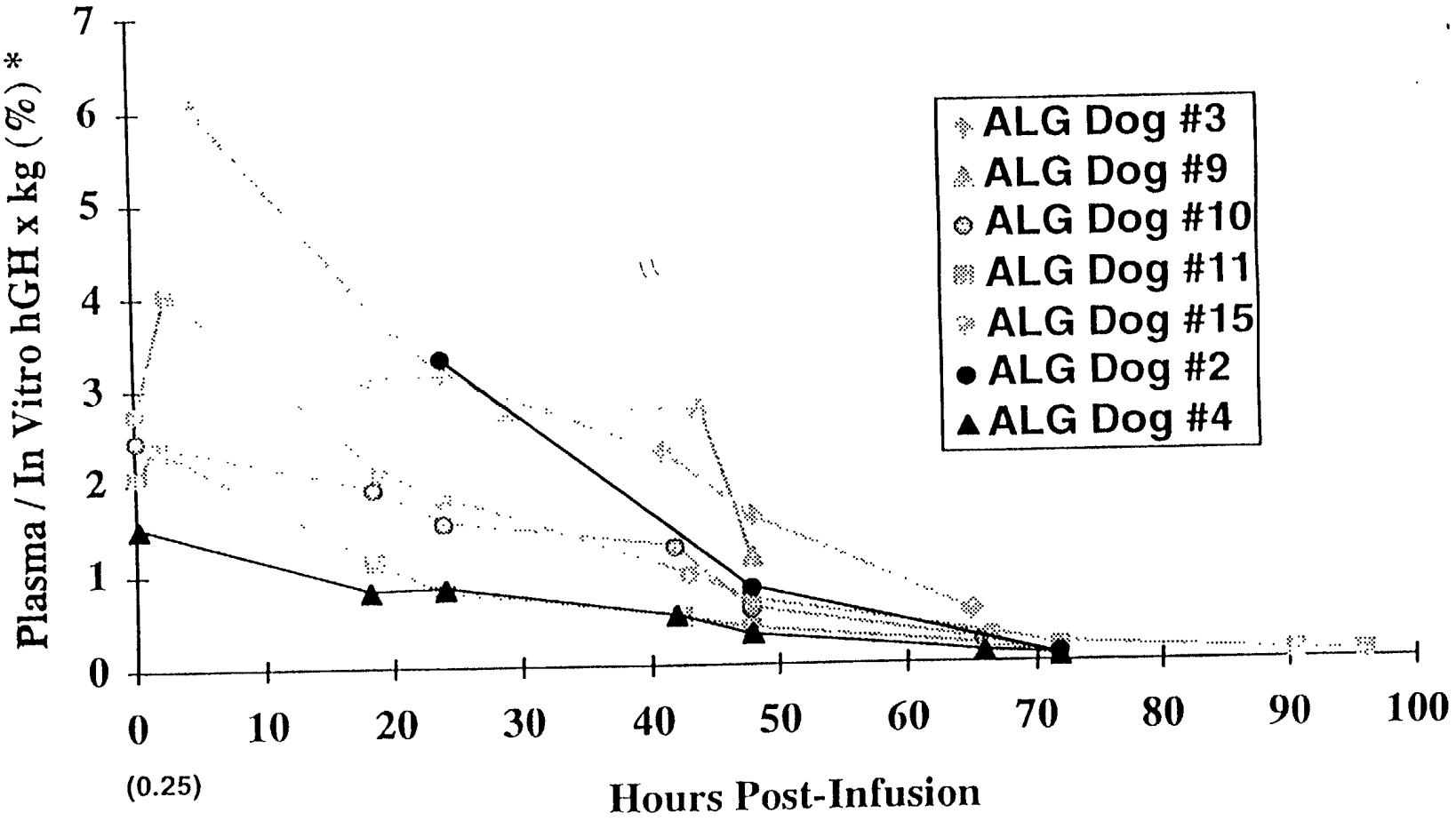
August 4, 2000
Date of Deposit

Signature

Matthew Morrissey
Typed or Printed Name of Person Signing Certificate

FIGURE 1



* Plasma (ng/ml) / In Vitro (total ug expression capacity / 24 hours) x kg Dog Weight x 100

FIGURE 2

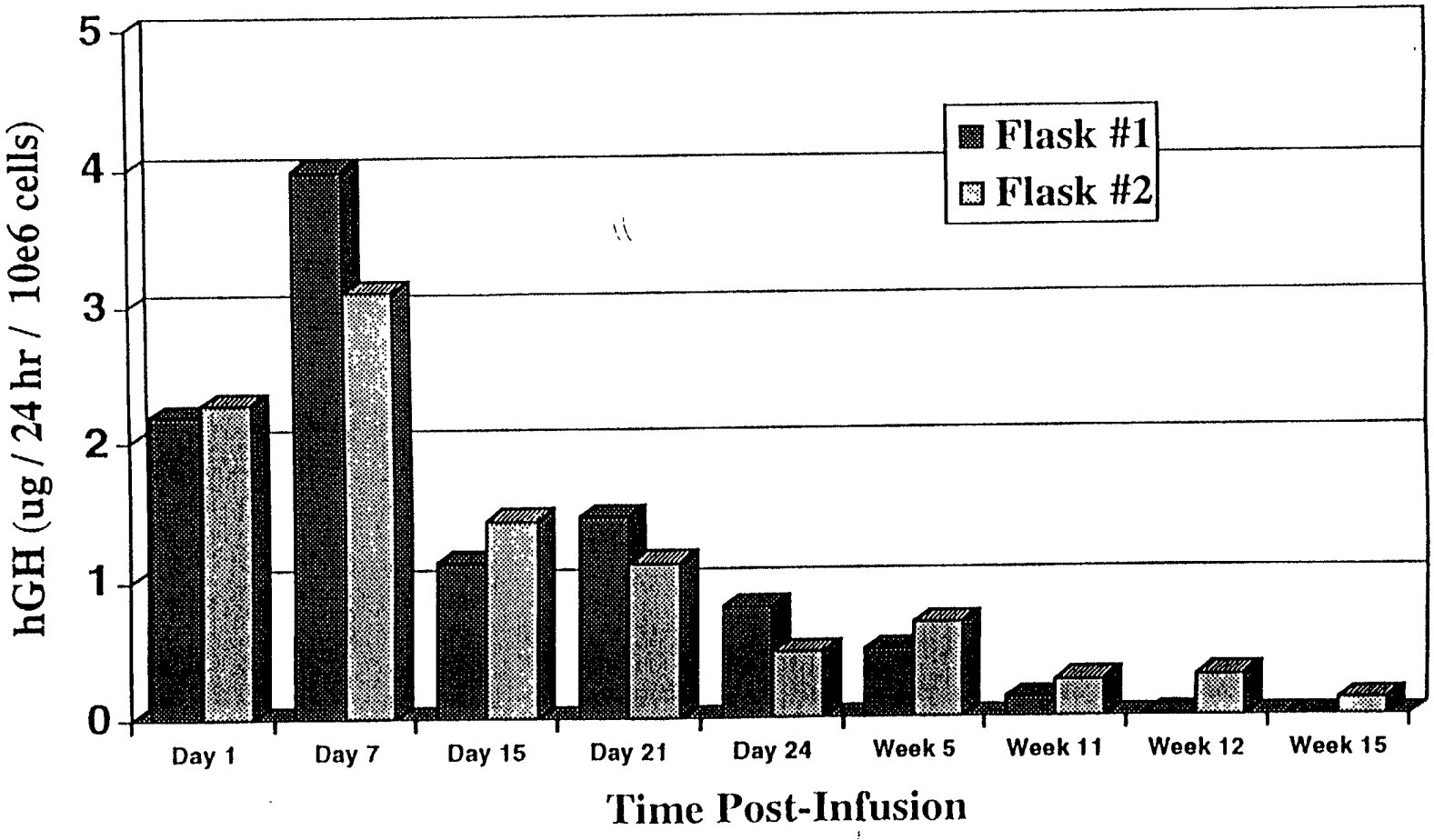
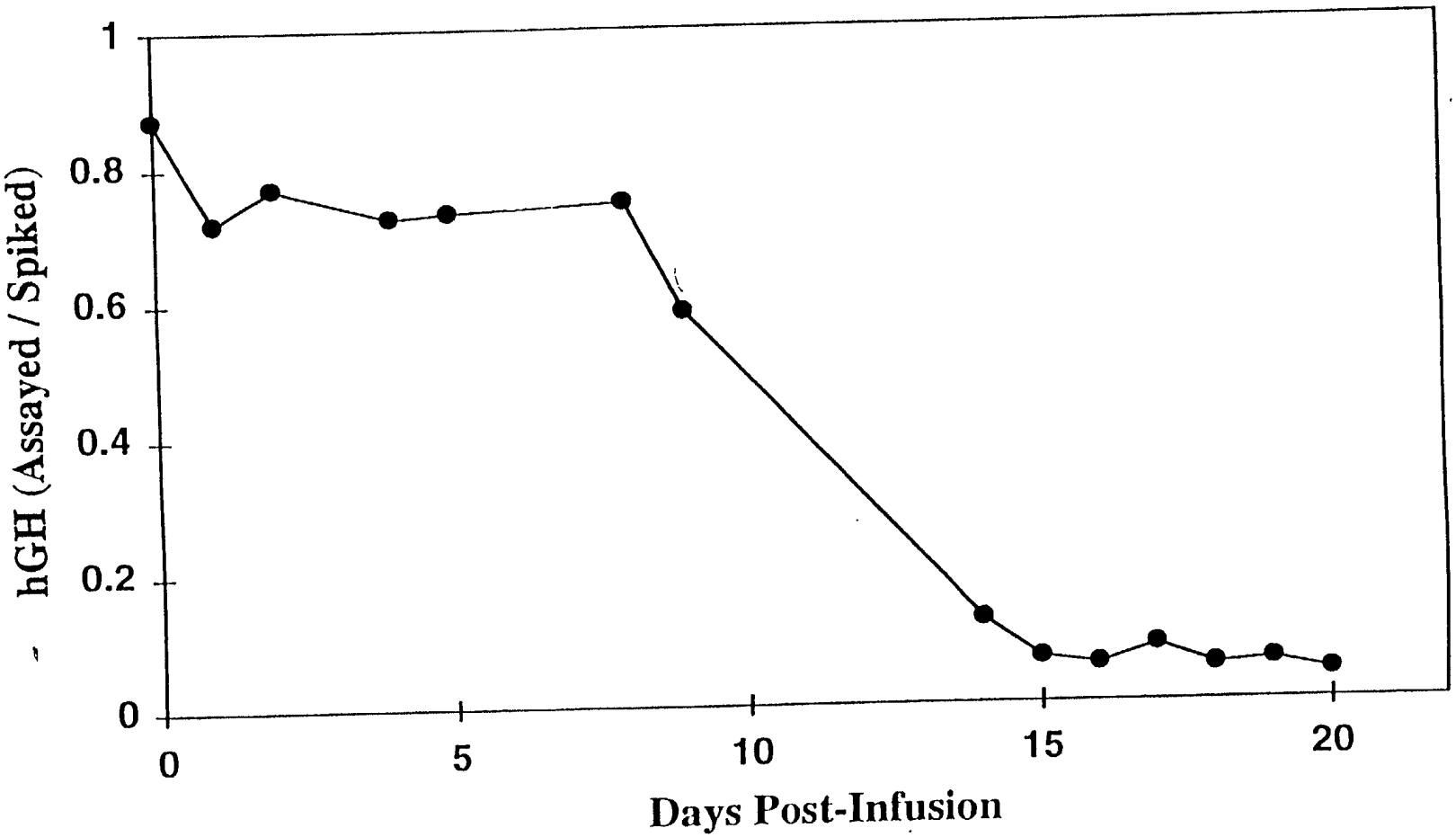
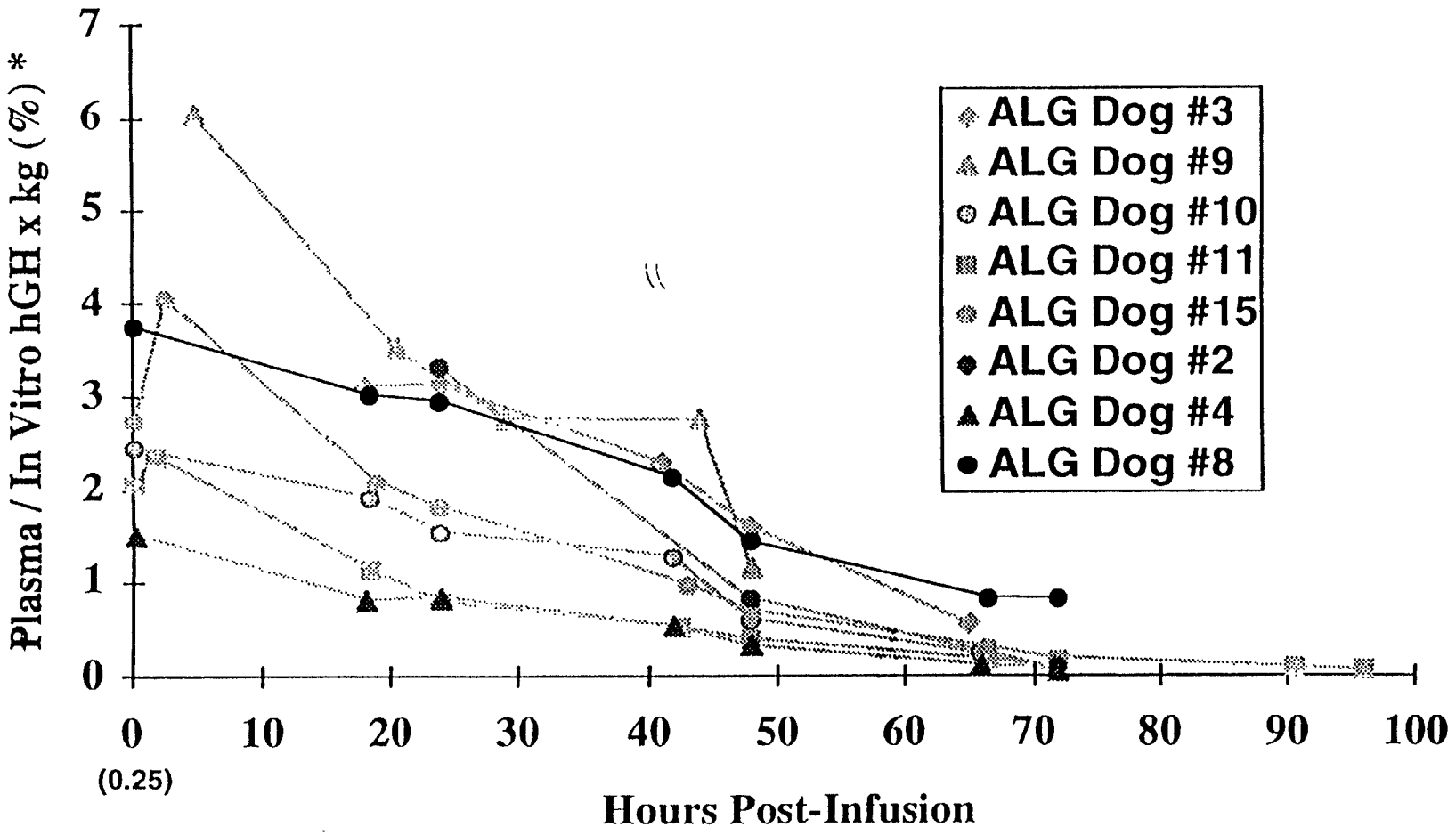


FIGURE 3



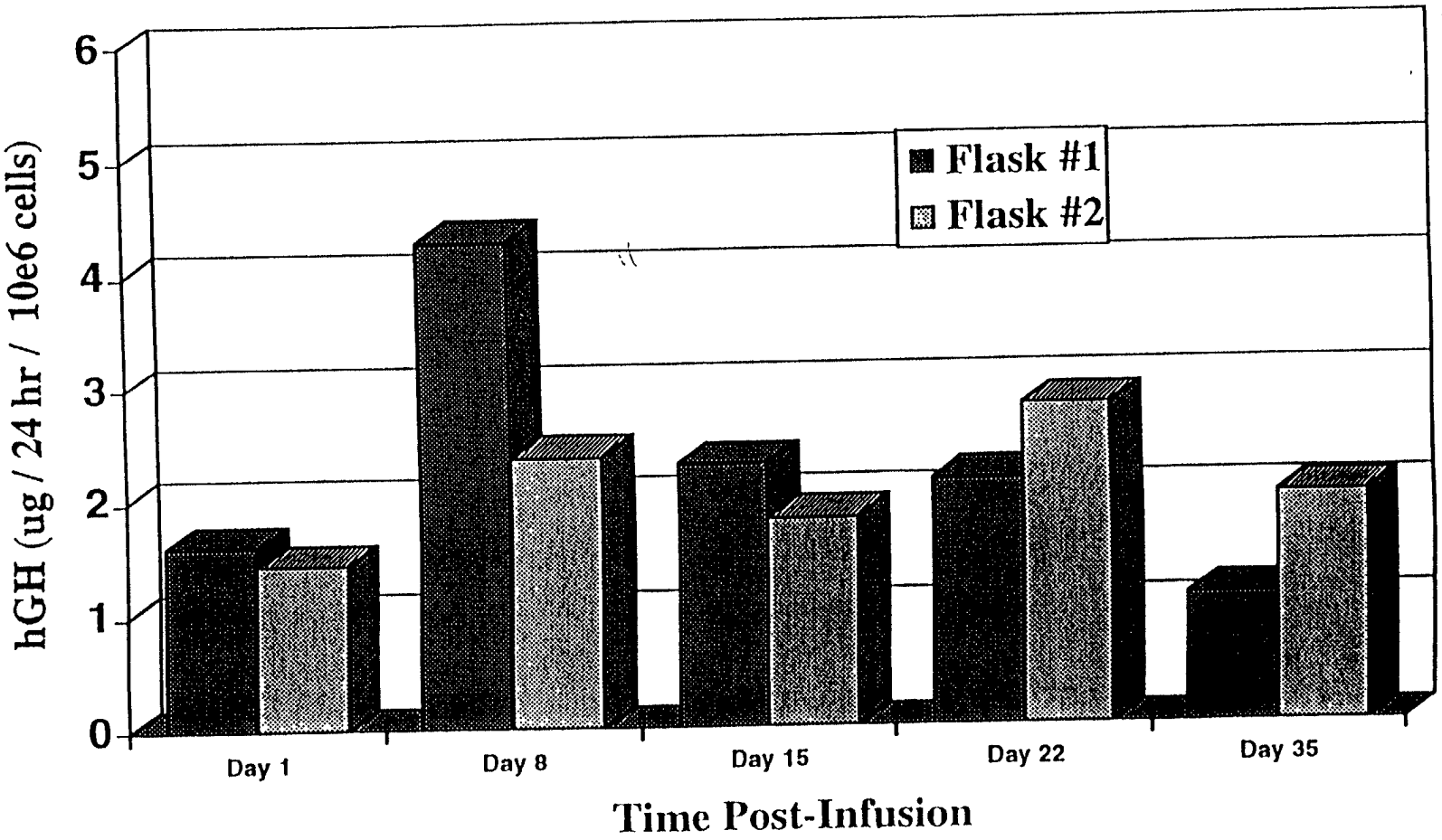
*O.5 ng/ml hGH spiked into all plasma samples.
All values adjusted for background levels of hGH within plasma for each plasma sample.

FIGURE 4



* Plasma (ng/ml) / In Vitro (total ug expression capacity / 24 hours) x kg Dog Weight x 100

FIGURE 5



COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled METHODS OF PREPARING BONE MARROW STROMAL CELLS FOR USE IN GENE THERAPY, the specification of which

☐ is attached hereto.

■ was filed on December 29, 1995 as Application Serial No. 08/581,053 and was amended on _____.

☐ was described and claimed in PCT International Application No. _____ filed on _____ and as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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